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p53 Tumor Suppressor Gene: Implications for Iatrogenic Cancer and Cancer Therapy

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INTRODUCTION

The p53 tumor suppressor gene has come to the forefront of cancer research because it is commonly mutated in human cancer and the spectrum of p53 mutations in these cancers is providing clues to the etiology and molecular pathogenesis of neoplasia [1–4]. Detection of p53 abnormalities may have diagnostic, prognostic, and therapeutic implications [5].

Recent studies investigating the mechanisms underlying the biological activity of p53 indicate that the protein is involved in gene transcription, DNA synthesis and repair, genomic plasticity, and programmed cell death [1–3,5–7]. These complex biochemical processes are performed by multicomponent protein machines. It is therefore not surprising that the p53 protein forms complexes with other cellular proteins (Fig. 1), and that some viral oncoproteins alter the functions of these machines by binding to p53 and perturbing its interactions with other cellular protein components (Fig. 2).

This brief review will discuss (a) the mutational spectrum of the p53 tumor suppressor gene and its potential use in identifying iatrogenic cancer and (b) the possible importance of the status of the p53 gene in responsiveness of human cancer to therapy.

p53 MUTATIONAL SPECTRUM ANALYSIS IN HUMAN CANCERS

The p53 gene is well-suited to mutational spectrum analysis for several reasons. First, since p53 mutations are common in many human cancers, a sizeable database has accrued whose analysis can yield statistically valid conclusions [8]. Its modest size (11 exons, 393 amino acids) permits study of the entire region, and it is highly conserved in vertebrates, allowing extrapolation of data from animal models [9]. Point mutations that alter p53 function are distributed over a large region of the molecule, especially in the hydrophobic midportion [1]. There, many base substitutions alter p53 conformation and sequence-specific transactivation activity; thus, correlations between distinct mutants and functional changes are possible. Frameshift and nonsense mutations that truncate the protein can be located outside of these regions, so evaluation of the entire DNA sequence yields relevant data. This situation differs from that of the ras oncogenes. Transforming mutations of ras occur primarily in three codons. They are thus limited to few sequence motifs that identify a critical functional domain [10]. The diversity of p53 mutational events permits more extensive inferences regarding mechanisms of DNA damage and mutation.

The mutational spectrum of the p53 in human cancers has been recently reviewed [4]. Therefore, only a few examples of associations between environmental carcinogens and specific p53 mutational spectra will be described.

Skin Carcinoma and Ultraviolet (UV) Light

A clear example of the value of mutational spectrum analysis in identifying carcinogen-specific mutations is seen in skin carcinomas, the most common type of human cancer, in which the role of UV light as the major carcinogen is unquestioned. Exposure to UV light increases the risk of both basal cell and squamous cell carcinoma [11,12], as well as the less common but more lethal melanoma. This physical mutagen produces distinctive pyrimidine dimers that, if unrepaired, can produce tandem mutations, most characteristically CC → TT transitions. Tandem dipyrimidine mutations are infrequently caused by mutagens other than UV light [13,14] and have rarely been observed in noncutaneous malignancies, i.e., 10 in over 2,400 tumors with p53 mutation. Thus, the observation that these tandem mutations are common in squamous cell (9%) and basal cell (12%) carcinomas of the skin directly incriminates both exposure to UV light as the cause of damage to the p53 gene, and the loss of its tumor-suppressor function in the development of the can-

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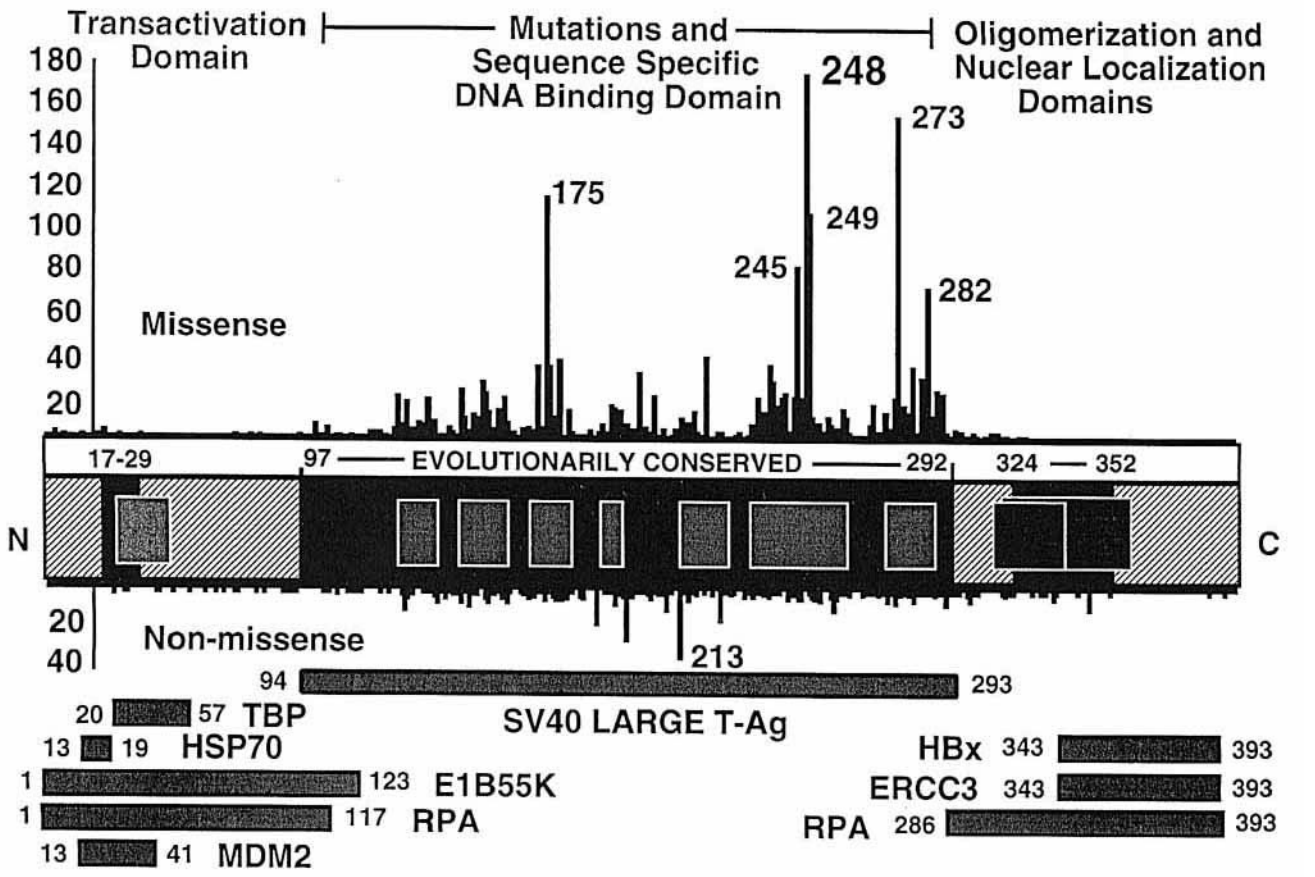

Fig. 1. Schematic representation of p53 molecule. The p53 protein consists of 393 amino acids with functional domains, evolutionarily conserved domains, and regions designated as mutational hotspots. Functional domains include the transactivation region (amino acids 20–42, diagonal-striped block), sequence-specific DNA binding region (amino acids 100–293), nuclear localization sequence (amino acids 316–325, vertical-striped block), and oligomerization region (amino acids 319–360, horizontal-striped block). Cellular or oncoviral proteins bind to specific areas of the p53 protein. Evolutionarily conserved domains (amino acids 17–29, 97–292, and 324–352; black areas) were determined using the MACAW program. Seven mutational hotspot regions within the large conserved domain are also identified (amino acids 130–142, 151–164, 171–181, 193–200, 213–223, 234–258, and 270–286, gray blocks). Functional domains and protein binding sites (white bars underneath) were compiled from references. Vertical lines above the schematic, missense mutations; lines below schematic, nonmissense mutations. The majority of missense mutations are in the conserved hydrophobic midregion, while nonmissense (nonsense, frameshift, splicing, and silent mutations) are distributed throughout the protein, determined primarily by sequence context.

cers [15]. The detection of rare cells with p53 mutations at dipyrimidine sites in sun-exposed non-malignant skin indicates that UV light initiated these mutations at the earliest stage of skin carcinogenesis [16].

Mutations at dipyrimidine sites in skin carcinomas show a nonrandom distribution among sites within the p53 gene. Of the 30 CC:GG dinucleotides (18 on the non-transcribed strand) in conserved domains, transitions are common (five or more) at only five (codons 151–152, 247–248, 178–179, 278, and 281–282); frequency of mutations also varies at other dipyrimidine sites (CT:GA, TT:AA, TC:AG). Recent work shows that rates of cyclobutane dimer repair vary among codons within p53 [17]. Comparison of the frequency of transition mutations to rates of repair shows that 94% of C:G → TT:AA transitions occur at sites where more than 10% of cyclobutane pyrimidine dimers remain 24 hours after UV treatment, suggesting that slow repair of these dimers is responsible for some of the site-specificity of UV-induced mutations [4].

Hepatocellular Carcinoma, Aflatoxin B_1, and Viral Hepatitis

The viral-chemical etiology and multistage pathogenesis of hepatocellular carcinoma (HCC) are archetypal of human carcinogenesis. It is estimated that 75–90% of HCC cases are attributable to hepatitis B virus (HBV) [18]. The relative risk of HCC is elevated in viral hepatitis carriers with chronic active hepatitis [19]. These findings suggest that cell proliferation and/or inflammatory response associated with chronic active hepatitis are the critical factors responsible for the increased probability of neoplastic transformation of the precursor cells of HCC. HBV DNA contains the X gene, which codes for a protein

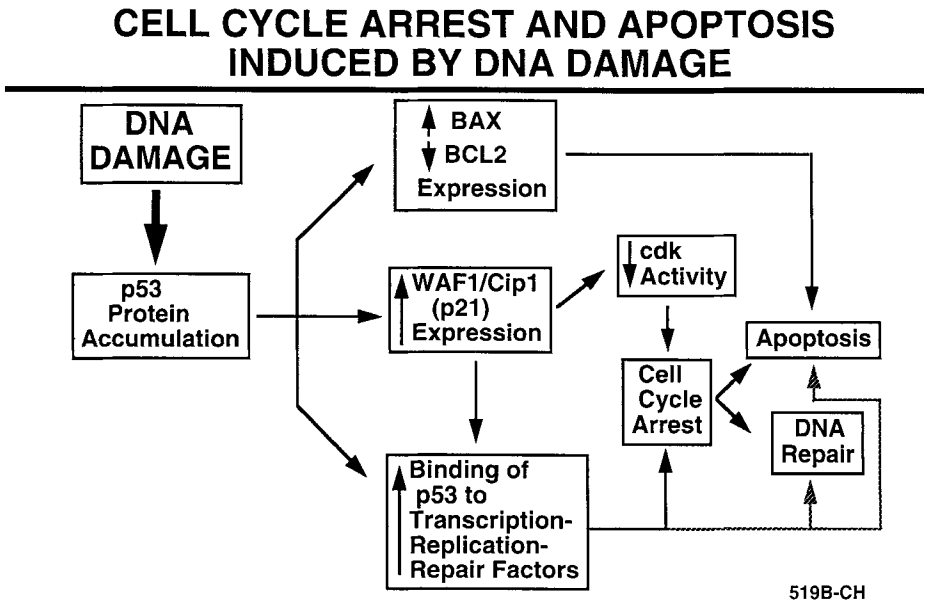

Fig. 2. DNA damage leads to p53 accumulation and subsequent changes in gene expression and protein-protein interactions.

(HBx) that modulates the transactivation of many cellular genes and is a candidate viral oncoprotein [20]. *Trans*genic mice containing the HBx gene in their germline have an increased frequency of HCC [21]. HBx protein binds with p53 in vitro and in vivo [22], inhibits p53 sequence-specific DNA binding and transactivation activities, partially disrupts p53 oligomerization, and prevents p53 binding to transcription-repair coupling factor XPB (ERCC3) [23]. These mechanisms of epigenetic p53 inactivation could account for the transactivation properties of HBx.

Alfatoxin B_1 (AFB) also is considered to be a significant etiological factor in certain geographic areas (e.g., southern Africa and Asia), where this mycotoxin is consumed in food contaminated by *Aspergillus flavis* [reviewed in 24]. Epidemiological studies conducted in the 1970s and 1980s provided statistical evidence that dietary consumption of AFB was positively correlated with incidence of HCC and suggested a synergistic interaction with alcoholic beverage consumption [25] or chronic active viral hepatitis [26–28].

Many investigators have reported data on the presence of chronic HBV infection and p53 mutations in the same tumors [29–37]. Their results indicate that HBV infection alone does not influence the rate of p53 mutation, and the AFB exposure is the most important influence on mutation prevalence (28% vs. 29% mutation prevalence in HBV positive vs. HBV negative cases in low AFB exposure areas; 54% prevalence in high AFB exposure areas, where almost all cases were HBV positive [29,32,37].

The results from a recent prospective cohort study of 18,244 people provide convincing evidence that AFB has an etiological role in hepatocellular carcinogenesis; they also indicate a synergy between HBV and AFB [38,39]. This nested case-control analysis shows statistically significant associations among the presence of AFB and its metabolites in urine specimens, serum HBV surface antigen positivity, and HCC risk. The finding of the promutagenic AFB-N^7-guanine adduct in the urine is further evidence that AFB has been activated to its electrophilic ultimate carcinogenic metabolite, AFB 8,9-oxide. Human hepatocytes in vitro can enzymatically activate AFB to its 8,9-oxide [40]; the interindividual variation in forming the AFB-N^7-guanine adduct may be 10-fold or greater [41]. Since several isoforms of cytochrome P450 enzymes can activate AFB [42], interpreting pharmacokinetic data for use in risk assessment will be complicated. The mutational spectrum of AFB [43,44] has been established in some experimental system: G:C → T:A transversions are the most common base substitutions [45–47]. The high frequency of AGG → AGT transversions on the nontranscribed strand at p53 codon 249 in HCCs from areas of China and Mozambique with a high HCC incidence could be due to the high mutability of the third base of codon 249 by AFB, and/or a selective growth advantage of hepatocyte clones carrying this specific 249^{ser} mutant in liver chronically infected by HBV.

The preferential mutability hypothesis has been tested by Cerutti and coworkers [48]. They found that in a human liver cell line exposed to AFB, the third base in codon 249 is preferentially but not exclusively mutated compared to immediately adjacent codons, suggesting that both preferential mutability and clonal selection are involved in hu-

man hepatocellular carcinogenesis. This highly sensitive and specific genotypic mutation assay also can be used to determine the p53 mutation load in non-tumorous liver tissue from donors living in geographic areas of high HCC incidence in which urinary AFB and/or macromolecular-AFB adducts have been detected. A pilot study using a genotypic mutation assay in non-tumorous liver tissues from HCC patients found that the loads of codon 249^{ser} mutations were elevated in specimens from Qidong, P.R.C., slightly elevated in one biopsy from urban Thailand, and did not exceed background levels in specimens from the U.S.A. [49]. These results indicate a positive association between loads of codon 249^{ser} mutation and dietary exposure to AFB. They also suggest that in some populations, p53 mutation occurs early in hepatocarcinogenesis and clonal expansion of these mutant cells occurs in the non-malignant tissue. A number of hypotheses could account for a selection advantage for these mutant cells, including altered responses to growth factors or resistance to programmed cell death induced by hepatitis viral infection. These and other hypotheses can be tested by in vitro models using human hepatocytes [40], and clinicopathologic studies such as correlation of aflatoxin-DNA adducts with codon 249 G:C → T:A transversions in individual patients.

Tobacco and Lung Cancer

Cigarette smoking is now thought to be responsible for 90% of lung carcinomas in men and 78% in women [50]. A large body of epidemiologic evidence has confirmed a dose-response relationship proportional to duration and amount of smoking [51,52]. p53 mutations are common in lung cancer, with the highest prevalence (70%) in small cell lung cancer (SCLC) and the lowest (33%) in adenocarcinomas. The prevalent mutation is G:C → T:A transversion with a predominance of guanine residues on the non-transcribed DNA strand, and the frequency of transitions at CpG sites (9%) is lower than in almost all other cancers. This spectrum is consistent with data for several different types of chemical carcinogens found in tobacco smoke. The highly mutagenic metabolites of poly aromatic hydrocarbons (PAHs) such as benzyl pyrene (BP) preferentially attack deoxyguanines and lead to mutations. One of the quantitatively minor adducts from the tobacco-specific N-nitrosamine NNK also leads to G:C → T:A transversions [53].

Analysis of p53 mutations in the database in relation to smoking history yields statistically significant conclusions consistent with the experimental data. The frequency of p53 mutation and of G:C → T:A transversion on the nontranscribed DNA strand are positively correlated with lifetime cigarette consumption [54]. Compared with non-smokers, smokers have significantly lower frequencies of all transitions 35 vs. 69%, $P < 0.05$), G:C → A:T transitions (21 vs. 69%, $P < 0.001$), and higher rates of G:C → T:A transversions with a DNA non-transcribed strand bias (29 vs. 0%, $P < 0.01$) [55]. These observations are consistent with the model of preferential repair of carcinogen-induced damage on the transcribed DNA strand [56,57]. Cigarette smoke and some of its components have been shown in vitro [58,59] to increase mutation frequency and cause defects in DNA repair which may contribute to the p53 mutation spectrum. These results also have implications in the current controversies concerning the relative risk of lung cancer in nonsmokers exposed to environmental (passive) tobacco smoke (ETS). Molecular analysis would contribute important data to the debate by determining whether critical mutations in lung cancers from these individuals show a spectrum similar to smokers (a dose-response increase in G:C→T:A transversions with exposure to ETS), although a large sample set may be required to detect changes in the spectrum.

Evidence exists of potential differences in pathways of carcinogenesis among histologic types of lung cancer. In vitro studies of lung cancer differentiation support a pluripotent cell of origin common to all types, or multiple cells which can differentiate by variable, often overlapping pathways [60–62]. Sufficient data exist to analyze the mutational spectra of squamous cell, large cell, adenocarcinoma, and small cell carcinoma (SCLC). p53 mutations are found in 70% of SCLC and 47% of NSCLC, including 65% of squamous, 60% of large cell, but only 33% of adenocarcinomas [4]. The mutational spectra are notable for an excess of A:T → G:C mutations in SCLC (11% vs. 6%), and an excess of deletions and insertions in NSCLC (14% vs. 4%). Subdividing NSCLS types reveals that the prevalence of G:C → T:A transversions in large cell, squamous, and small cell carcinomas is similar (43–49%), but they are less common in adenocarcinoma. This finding is consistent with the weaker association of adenocarcinoma with cigarette smoking [63–65], since G:C → T:A transversions are thought to result from bulky molecules found in tobacco smoke. In adenocarcinomas, higher proportions of G:C → A:T (non-CpG site) and G:C → C:G mutations are seen, suggesting the presence of other carcinogens. Since adenocarcinomas are the most common cell type in women, and squamous cell is less frequent, analysis of mutational spectrum by gender (controlling for cell type and smoking history) may provide clues to different carcinogenesis pathways, or differing susceptibilities to tobacco-associated carcinogens.

IATROGENIC CANCER

Advances in cancer therapy, particularly treatment of pediatric cancers, have produced long-term survivors. Ironically, these survivors have an increased risk of developing a second cancer (termed iatrogenic, or treatment-related) which may be linked to genotoxic effects of the initial chemotherapeutic agents [66,67]. Prominent

examples include: (i) leukemia, non-Hodgkin's lymphoma, and solid tumors, principally lung cancer, following Hodgkin's disease; (ii) lung cancer following radiation therapy for breast cancer; (iii) hepatic angiosarcoma following Thorotrast (thorium dioxide) administration as a radiographic contrast agent [68–71]; (iv) leukemia and bladder cancers following cyclophosphamide therapy for non-Hodgkin's lymphoma; (v) hepatocellular carcinoma related to oral contraceptive use; and (vi) tamoxifen-associated endometrial cancer following breast cancer therapy. Examples will be further discussed to illustrate three classes of therapeutic agents: radiation, chemotherapy, and hormonal agents.

Radiation Therapy

Hodgkin's disease. Radiation therapy, chemotherapy, or combination therapy have produced long-term survivors of Hodgkin's disease [72]. Acute non-lymphocyte leukemia, non-Hodgkin's lymphoma, and lung cancer are the most common cancers that develop following treatment for Hodgkin's disease [73–75]. Some studies have suggested that development of leukemia is more closely associated to chemotherapy than to radiation therapy. Vincristine sulphate, prednisone, and the alkylating agents mechlorethamine, procarbazine, and cyclophosphamide are commonly used to treat Hodgkin's disease; these agents may cause a 5–10% increased risk of acute non-lymphocyte leukemia 10 years after therapy [76,77]. Other investigators found an increased risk of lung cancers among patients who received radiotherapy and/or chemotherapy, but failed to demonstrate a role of chemotherapy [78,79]. Although these clinical treatments contribute significantly to the development of secondary malignancies, additional factors including smoking and genetic predisposition play important roles.

Radiation therapy for breast cancer. Lung cancer following radiation therapy for breast cancer is another example of iatrogenic cancer [68,80–82]. Inskip and collaborators found that in a cohort of women who received radiotherapy for breast cancer before the 1970s, the relative risk to develop lung tumor was 1.8 ten years after radiation treatment and increased to 2.8 when the follow-up was 15 or more years [80]. Furthermore, the risk of lung cancer is higher among women who smoke, suggesting a multiplicative effect of radiation therapy and smoking [81].

Chemotherapy

Cyclophosphamide and bladder cancer. Cyclophosphamide (CTX) is used to treat several cancers including lymphoma, myeloma, leukemia, neuroblastoma, ovarian cancer, retinoblastoma, and breast cancer. It is metabolized to an alkylating agent which cross-links DNA and to the unsaturated aldehyde, acrolein. An association between CTX therapy and bladder cancer has been noted since the late 1970s [83–85]. To further investigate mechanisms of CTX-induced bladder cancer, 19 bladder cancers from non-Hodgkin's lymphoma patients treated with CTX are being analyzed for p53 mutations (Soini et al., in preparation). The resulting mutation pattern will be compared to tobacco-associated bladder cancers [86]. The tobacco-associated mutation pattern includes: (i) predominance of G → C transitions (26% overall); (ii) six identical point mutations at codon 280; (iii) 5/15 tumors containing point mutations from cigarette smokers had double mutations, four of which were tandem mutations on the same allele; and (iv) absence of G → T transversions which are associated with bulky chemical carcinogens (i.e., tobacco smoke). It is possible that such analyses will define the mutation spectrum of CTX in human epithelial cells.

Hormonal Agents

Oral contraceptives and liver cancer. In the last 20 years, animal models and epidemiological studies have implicated oral contraceptives in liver cancer development. Oral contraceptives are combinations of synthetic estrogens; the most commonly used are ethinyl estradiol and mestranol [87]. Both hormones promote hepatocarcinogenesis in rodents [88,89]. Moreover, ethinyl estradiol alone stimulates DNA synthesis several-fold in cultured rat hepatocytes suggesting that this estrogen has direct growth-related effects [90]. Additional experiments in rat hepatocytes have shown that ethinyl estradiol dramatically increases the activity of EGF (Epidermal Growth Factor) on DNA synthesis and also increases the number of EGF receptors per cell [90].

Among women taking oral contraceptives, mainly among long-term users, a significant increase of liver damage has been observed. Oral contraceptives are strongly related to development of benign liver adenomas [91,92] and focal nodular hyperplasia [93]. Furthermore, a few histologic studies have shown a transition from adenoma to carcinoma in women who used oral contraceptives [94]. Moreover, a significant association between oral contraceptives and hepatocellular carcinoma has been shown by numerous studies [95,96]. They have also shown that the risk of developing liver carcinoma increases with duration of use of oral contraceptives. For example, Forman and collaborators [97] found that after 8 years or more of oral contraceptive consumption, the risk increased from 3.8 ($P < 0.05$) to 20.1 ($P < 0.01$). Yu et al. [96] reported that the relative risk of hepatocellular carcinoma was 3.0 and after 5 years of oral contraceptive use, the relative risk was 5.5. Although additional factors, including HBV/HCV infection, cigarette smoking, alcohol consumption, and the use of other estrogens, are linked to the development of hepatocellular carcinoma, the risk remained unchanged after adjustment for these factors [96]. Most of the investigations, however, concerned pop-

ulations in which HBV/HCV viral infection had a low frequency, and alfatoxin B_1, another liver cancer risk factor is undetectable.

Tamoxifen and endometrial cancer. Tamoxifen is an antiestrogenic compound used in hormonal therapy for breast cancer. While it usually inhibits breast cancer growth, it may have estrogenic activity in other tissues. An increased rate of endometrial cancer observed among patients treated long-term has been attributed to estrogenic activity [98–100]. Mechanistic studies attempt to relate estrogen and tamoxifen activities to proto-oncogenes, growth factors, growth factor receptors, and cell cycle regulation [101,102]. A prospective breast cancer chemoprevention trial using tamoxifen has begun; the goal is to recruit 16,000 healthy pre-menopausal women [103,104]. In multiple, randomized clinical trials using tamoxifen in breast cancer patients, the cumulative frequency of infiltrating endometrial cancer in women receiving tamoxifen was 0.5% compared to 0.1% in the control group [105]. These statistics indicate that additional tamoxifen-associated endometrial cancers will occur.

p53 Mutational Spectrum Analysis of Iatrogenic Cancer

Lung cancer following radiation therapy for Hodgkin's disease. A goal of p53 mutational analysis is to elucidate disease mechanisms. The etiology of lung cancer following radiation treatment for Hodgkin's disease is an appropriate area of investigation, since multiple carcinogens make contributions, including radiation and oxidative damage, chemotherapy, and tobacco smoking in many patients. The relative contributions of each are unknown currently. Analysis of patterns of p53 mutations and correlations with treatments and exposures may provide clues to such questions.

A recent analysis of lung tumors which developed in 11 male smokers following radiation therapy for Hodgkin's disease produced evidence consistent with a model in which radiation therapy acted as an initiator, and smoking served as a promoter [106]. There were a total of six mutations in tumors from five patients; there were four G:C → G:C and one A:T → C:G transversion. Despite moderate to heavy smoking histories in all patients, there were no G:C → T:A transversions which are closely associated with tobacco smoke carcinogens [1,4,55]. The prominence of G:C → A:T transitions is characteristic of both radiation and oxidative damage [107–115]; however, only one transition occurred at a CpG site, and this suggests that radiation, instead of oxyradical enhanced deamination of 5-methylcytosine, was the primary mutagen. If confirmed, these results indicate that chemoprevention with retinoids and/or smoking cessation could reduce the rate of secondary lung cancer development in these patients. Although the sample set is too small to provide definitive results, the data indicate that well-designed studies of p53 mutational analysis can provide insight into disease mechanisms.

CANCER THERAPY

Rapidly dividing cancer cells have long been considered to be the targets of chemo- and radiotherapy. This dogma has influenced screening of compounds for anticancer activity and design of therapeutic regimens for use in the clinic. Recent clinical and laboratory evidence has prompted a reevaluation of this paradigm and has emphasized the importance of apoptosis (programmed cell death) in response to treatment.

Apoptotic death is a normal cellular phenomenon characterized by specific biochemical and morphological criteria [116], and aberrations in this pathway are important in carcinogenesis [117]. Multiple endogenous and exogenous inducers of apoptosis have been identified, including cytokines and cytotoxic drugs. These agents transmit their signals through at least two poorly understood biochemical pathways, one of which depends on wild type p53 protein [118,119]. In some human and murine tumor cell lines, introduction of wild type p53 triggers apoptosis [120–123]. DNA damage, especially double strand breaks, initiates the p53-dependent pathway (Fig. 2). Putative mechanisms include transcriptional control of specific genes (e.g., $p21^{WAF1}$ and bax) and protein-protein interactions with multicomponent protein machines involved with DNA replication and repair.

This property of wild type p53 suggests an extension of the model of p53 as a regulator of genomic stability. p53 is known to mediate the G_1 checkpoint of the cell cycle [124]. In cells with wild type p53, DNA damage-induced p53 expression leads to growth arrest, during which DNA repair might occur. The observation that p53 binds to DNA repair proteins [125] may suggest a mechanism for coupling of these processes. If repair is inadequate, wild type p53 may then trigger apoptotic death. In cells with loss of p53 function due to mutation, this growth arrest and cell death would not occur. These cells therefore have a growth advantage, and are more likely to produce daughter cells with additional DNA damage.

The hypothesis that tumors containing wild type p53 protein are more responsive to ionizing radiation and cytotoxic drugs is supported by direct laboratory data and some inferences derived from clinicopathological findings in human cancers. Ionizing radiation and cancer chemotherapeutic agents such as doxorubicin, etoposide, and cisplatin produce DNA damage and induce apoptosis in sensitive cells [126]; levels of p53 protein rise in cells experiencing such damage [127]. In vitro studies of murine cells indicate that the presence of wild type p53 protein is necessary for apoptosis to occur in response to

these agents, whereas it is not required for thymocyte apoptosis induced by glucocorticoids [118,119,128].

The relationship between p53 function and response to chemo- or radiotherapy has not yet been directly assessed in human cancer in vivo. Presence of p53 protein detected by immunohistochemistry often indicates stabilization of the protein by mutation. Positive staining for p53 (suggesting mutation) correlates with poor prognosis in several cancer types [129]; differentiated response to therapy may play a role. Some tumor types that rarely contain p53 mutations, such as Wilms' tumor and testicular carcinoma, are generally sensitive to chemo- and radiotherapies. However, melanomas, a tumor type notoriously resistant to therapy, also usually contain wild type p53, and small cell lung cancers usually contain mutations but are frequently radio- and chemosensitive. Thus, the relationship between p53 status and in vivo behavior promises to be complex.

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